(C=O of *N*-acetyl) and 25.5 (CH_3 of *N*-acetyl). CM-chitin sodium salt: ds 0.7–0.8 for CM; $[\alpha]_D^{18}$ −4–−6° (*c* 1.0, aqueous 0.5% sodium hydroxide solution); ν_{max}^{KBr} 1660 CO_2^- and C=O) of *N*-acetyl; ^{13}C NMR (D_2O): δ 180.0 (C=O of CM), 177.5 (C=O of *N*-acetyl), and 25.5 (CH_3 of *N*-acetyl). CM-chitin: ds 0.7–0.8 for CM; $[\alpha]_D^{18}$ −4° (*c* 0.6 water); ν_{max}^{KBr} 1740 (C=O of CO_2H), and 1660 and 1560 cm^{-1} (C=O and NH of *N*-acetyl).

[46] Isolation of Oligomeric Fragments of Chitin by Preparative High-Performance Liquid Chromatography

By KEVIN B. HICKS

Chitin, a biopolymer composed of β-1,4-linked 2-acetamido-2-deoxy-D-glucose (GlcNAc) residues, is commonly found in the cell walls of fungi and in the exoskeletons of most arthropods. The natural polymer is quite insoluble in water, but partial acid hydrolysis yields a series of soluble β-1,4-linked oligomers that are useful substrates for studies on enzymes (i.e., lysozyme) and that have also been reported[1] to be useful in a variety of medicinal and industrial applications. Large amounts of chitin-containing biomass exist today in the form of shellfish processing wastes. The conversion of these abundant by-products into useful oligomers and derivatives is an important current research goal.

Pure chitin oligomers are usually prepared by the fractionation of acid-hydrolyzed chitin on large columns packed with charcoal,[2] or gel filtration media.[3] Although these techniques produce relatively pure standards, they are tedious and quite time consuming; some separations require more than a week. Recently,[4–6] the technique of high-performance liquid chromatography (HPLC) has been used to separate, on the analytical scale, oligomers from chitin hydrolysates. In this report, we demonstrate the separation of chitin fragments on a variety of HPLC stationary phases, including aminopropyl silica, octadecyl silica, and cation-exchange resins in the Ag^+ and H^+ form, and apply these principles to the preparative HPLC fractionation of milligram to gram quantities of chitin oligomers with degree of polymerization (dp) values of 2–6.

[1] R. A. A. Muzzarelli, "Chitin." Pergamon, Oxford, 1977.
[2] S. A. Barker, A. B. Foster, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, p. 2218 (1958).
[3] B. Capon and R. L. Foster, *J. Chem. Soc.*, p. 1654 (1970).
[4] P. van Eikeren and H. McLaughlin, *Anal. Biochem.* **77**, 513 (1977).
[5] S. J. Mellis and J. U. Baenziger, *Anal. Biochem.* **114**, 276 (1981).
[6] K. Blumberg, F. Liniere, L. Pustilnik, and C. A. Bush, *Anal. Biochem.* **119**, 407 (1982).

Preparation of Chitin Hydrolysate

Chitin (Pfanstiehl Laboratories, lot 11144) is ground in a mortar grinder (Brinkman Instruments,[7] Model RMO) to a fine powder, then hydrolyzed as follows. Chitin powder (50 g) is added with constant stirring, to 200 ml cold (4°) concentrated HCl in a flask immersed in an ice bath. The flask is stirred at room temperature for 2.5 hr, warmed to 40°, and then stirred for an additional hour. After cooling, 2700 ml of Duolite A-561-free base form (Diamond Shamrock Corporation), slurried in 1 liter of water, is slowly added to neutralize (pH >3) the acid. The solution is filtered through Whatman 1 paper covered with Celite (Johns Manville) and then reduced in volume to 250 ml by evaporation at reduced pressure and low temperature (<30°). Free amino groups on the chitin fragments are re-N-acetylated[2] by adding methanol (22.5 ml) and acetic anhydride (6 ml) to the cold (4°) 250 ml filtrate in the presence of 270 ml of Amberlite (Rohm and Haas) IRA-400, carbonate form. After stirring the reaction for 1.5 hr at 4°, the solution is again filtered and deionized by passage through columns containing 200 ml Amberlite IR-120 H^+ and 50 ml Duolite A-561 free base form, respectively. The effluent and wash from the final column is evaporated carefully to 150 ml, then lyophilized. The yield is 9.41 g of white fluffy solid.

Chromatographic Equipment

HPLC is performed on a Gilson gradient HPLC system composed of two model 303 solvent pumps, an Apple IIe controller, a Rheodyne model 7125 fixed loop injector, a Kratos 520 column heater, and a Waters model 403 preparative differential refractometer. For automated injections, a Gilson model 302 pump, controlled by the microprocessor, is used to inject samples. Fractions are collected manually. Chromatograms are recorded on a Houston Instruments recorder. All solvents (HPLC grade) are filtered prior to use and degassed with helium during chromatography.

High-performance cation exchange columns (0.78 × 30 cm) in three ionic forms, HPX-87-H (H^+ form), HPX-87-C (Ca^{2+} form), and HPX-42-A (Ag^+ form), are purchased from Bio-Rad Laboratories. The former two columns are packed with <10-μm, 8% crosslinked, sulfonated

[7] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

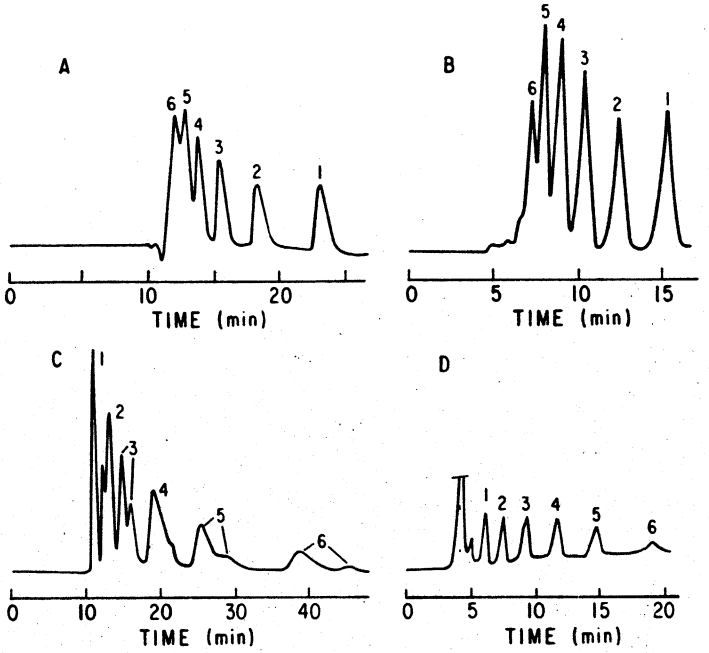

FIG. 1. Comparative separations of chitin oligomers on four HPLC stationary phases. (A) HPX-87H at 75°; mobile phase: 0.01 *N* H_2SO_4 at 0.3 ml/min, 20-μl (1 mg) injection. (B) HPX-42A at 75°; mobile phase: H_2O at 0.6 ml/min, 20-μl (1 mg) injection. (C) Dynamax C-18 at 25°; mobile phase: H_2O at 4 ml/min, 500-μl (35 mg) injection. (D) IBM Amino at 25°; mobile phase: acetonitrile (75)/H_2O (25) at 1 ml/min, 20-μl (0.4 mg) injection. Refractive index detection at various attenuations. Numerals above peaks refer to dp values.

polystyrene. The latter column is packed with 4% crosslinked resin. The analytical (0.46 × 25 cm) aminopropyl silica (5 μm) column is purchased from IBM Instruments. The Dynamax C-18 silica column and the Dynamax custom-packed aminopropyl silica column are obtained from Rainin Instrument Company, and are packed with 7-μm irregular silica into preparative sized (2.0 × 30 cm) axial compression cartridges. Precolumns are used with all columns except the Rainin preparative models.

Comparative Separations on Four HPLC Stationary Phases

Figure 1 shows the analytical scale separation of chitin oligomers on four commercially available stationary phases. On high-performance cation-exchange resins in the H^+ (Fig. 1A) and Ag^+ (Fig. 1B) forms, the oligomers are separated by a combination of size-exclusion, hydrophobic interaction, and ligand-exchange mechanisms,[8] resulting in an elution

[8] K. B. Hicks, Lim, and M. J. Haas, *J. Chromatogr.* **319**, 159 (1985).

order that follows descending molecular weight. These columns must be operated at ≥75° in order to obtain narrow, resolved peaks. It is noteworthy that the more commonly used Ca^{2+} form of these resins was not a useful stationary phase for this separation; poor resolution resulted under all conditions tested. Complete separation of all six oligomers was also accomplished on a C_{18} bonded silica (reversed-phase) column (Fig. 1C), but as previously noted,[6] peaks are broad, due to the separation of the α- and β-anomeric forms of each oligosaccharide. The aminopropyl silica (normal phase) column (Fig. 1D) provides excellent resolution of the oligomers, completely separating dp 1 through 6 in less than 20 min. On both of the silica-based stationary phases (Figs. 1C and D), chitin oligomers elute in an opposite (and complementary) order to that seen on the cation-exchange resins. In all of the chromatographic systems tested here, each peak appeared to contain only one component, and pure GlcNAc and *N,N'*-diacetylchitobiose coeluted with peaks 1 and 2, respectively, in Fig. 1A–D.

Preparative HPLC of Chitin Oligomers

Each of the stationary phases described above can be used for isolation of quantities of purified chitin fragments. The cation-exchange column in Fig. 1A, is normally used as an analytical column, but its relatively large diameter (0.78 cm) allows it to be used for semipreparative HPLC (Fig. 2A), in which 15 mg of the crude mixture may be fractionated. The

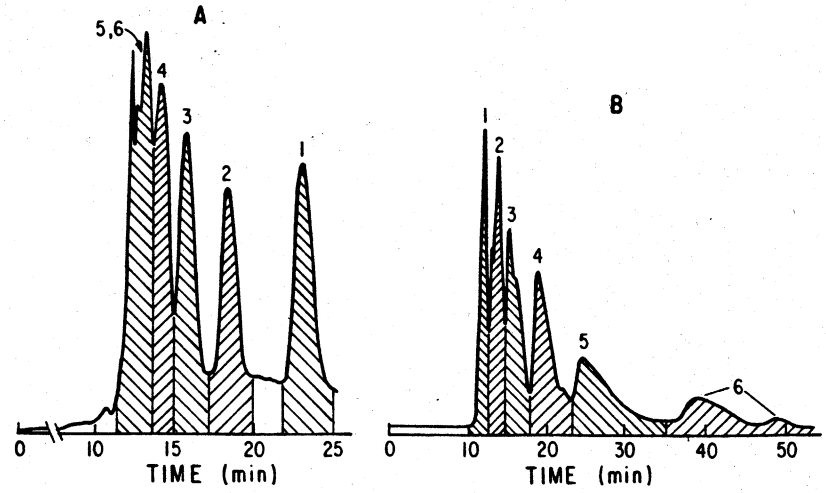

FIG. 2. Preparative HPLC of chitin fragments. (A) HPX-87H column, 200-μl (15 mg) injection; other conditions as in Fig. 1A. (B) Dynamax C-18 column, 2[illegible] μl (140 mg) injection; system pressure: 300 psi, other conditions as in Fig. 1C.

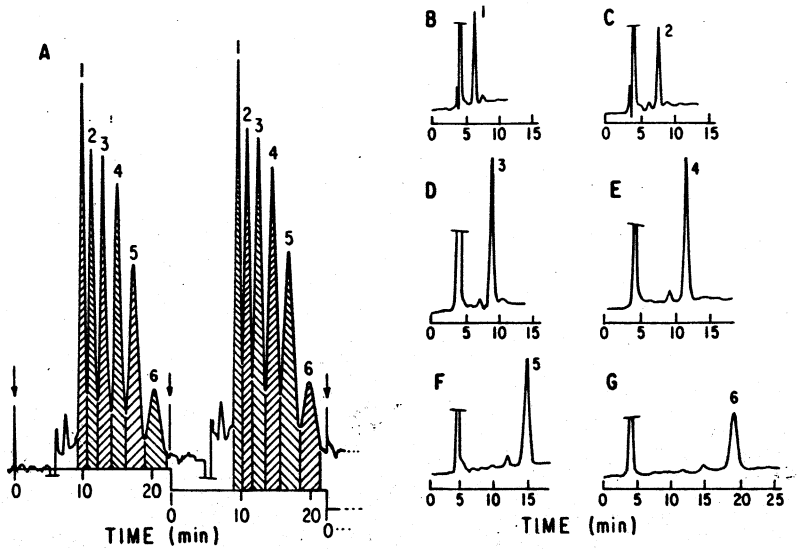

FIG. 3. Preparative HPLC of chitin fragments on aminopropyl silica, using automated, repetitive injections (injections shown by arrows). (A) Sequence of two automated injections (3.5 ml volume, 100 mg) on Dynamax custom-packed NH_2 column, mobile phase: acetonitrile (60)/H_2O (40), at 12 ml/min and 1500 psi. (B–G) Purified fragments collected from A, analyzed by the column described in Fig. 1D.

HPX-42A column gave good analytical separations, but developed high back pressures after several semipreparative-sized injections. Larger sample sizes (100–140 mg) can be injected onto the preparative C_{18} bonded phase column (Fig. 2B) and the preparative aminopropyl silica column (Fig. 3A).

Discussion

Each phase demonstrated in Figs. 2 and 3 has unique characteristics, as shown in Table I. The H^+ form column is quite durable and inexpensive to operate, yet is only useful for isolating milligram amounts of oligomers. The C_{18} column has much greater sample capacity, and can be used to fractionate gram quantities of chitin oligomers. This column must occasionally be washed with 50% acetonitrile to restore its capacity, but otherwise is exceptionally durable in this application. Because this column is operated with inexpensive mobile phase (H_2O), at low flow rates and low back pressures, it can be easily accommodated on standard analytical HPLC systems. However, as with the cation-exchange column, some of the isolated oligomers are only 54–75% pure (Table I), and must be rechro-

TABLE I
PREPARATIVE HPLC OF CHITIN OLIGOMERS ON THREE HPLC STATIONARY PHASES

Column[b]	Sample size (mg)	Analysis time (min)[c]	Milligrams of each oligomer collected per injection (% purity)[a]					
			1	2	3	4	5	6
HPX-87H	15	20	1.8 (95)	1.6 (74)	1.7 (72)	1.6 (75)	2.7	
Dynamax C-18	140	40	15.0 (95)	27.0 (75)	29.0 (57)	29.0 (54)	23.0 (61)	15.0 (95)
Dynamax NH_2	100	23	12.0 (92)	12.0 (92)	15.0 (94)	16.0 (94)	15.0 (94)	8.0 (98)

[a] Purity was determined by analytical HPLC on the basis of peak area.
[b] Chromatographic conditions as in Figs. 2 and 3.
[c] Time needed between repetitive injections.

matographed, individually, to give oligomers with about 95% purity. In contrast, fractions from the aminopropyl silica column are from 92 to 98% pure from a single chromatographic run (Table I); a second purification gives oligomers that are >98% pure. Samples can be processed rapidly (every 23 min) on this column and it is possible to use automated, repetitive injections as shown in Fig. 3A, to fractionate 1 g of chitin hydrolysate in about 270 min. The resulting fractions (Fig. 3B–G) contain between 80 and 160 mg of oligomers at purity levels averaging 94%. A disadvantage of this column is its relatively short lifetime,[9] which is somewhat offset by carefully pretreating samples prior to injection, and following other recommended[9] precautions (see Note Added in Proof). In addition, the requirements for binary mobile phases and higher (12–15 ml/min) flow rates can preclude the use of these columns on some analytical HPLC systems. Surprisingly, the operating back pressure of this column was no greater than that of an analytical column packed with the same phase.

When oligomer fractions from these columns are concentrated, extreme care must be taken to use the most gentle conditions available. The fractions from the cation-exchange column must also be treated with anion-exchange resin, to remove sulfuric acid. Fractions from the aminopropyl column should be evaporated under diminished pressure, and at low temperatures (<30°) to remove acetonitrile. The resulting aqueous fractions from all columns should then be carefully freeze dried, rather than evaporated to dryness. The latter process yields aggregated oligomers which do not redissolve into aqueous solution. Even under the best of conditions, the lyophilized powders will tend to aggregate, and levels of insoluble, aggregated material increase with storage time. Pure fractions should be stored at low temperatures (−20°) prior to use.

In summary, preparative HPLC on commercially available stationary phases can be used to efficiently fractionate gram quantities of oligomers from chitin hydrolysates. The choice of column depends on the amounts of oligomers required, the purity desired, and on the supporting instrumentation that one has available.

Acknowledgment

I acknowledge Scott M. Sondey for excellent technical assistance.

Note Added in Proof

An improved version of the Dynamax NH_2 column is now available. This column is extremely durable and may last approximately 10× as long as previous aminopropyl silica gel columns. This fact makes the use of this column quite practical.

[9] B. Porsch, *J. Chromatogr.* **253**, 49 (1982).

[4

By

Isolation of C

Principle

In crustace
salts, such as
Therefore, in
three steps ar
(step 1a) or wi
teinization wi
proteolytic act
organic solven
above-mentio
which deprote

For the pu
which consists
causes some c
tion, on the
consists of ste
chitin, althou
(Table I).

Startin

Shells of cr
shells are scra

[1] K. Shimahara,
Chitosan, and
1984.
[2] Some investiga
levels of unrem
[3] M. Takeda and
[4] M. Takeda and
[5] A. W. Bough, V
(1978).
[6] C. J. Brine and
[7] R. H. Hackman
[8] J. N. BeMiller a